

Rule 131 Petition -- AN 09/472,558

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Electroporation of pWTC1 (old sample) into Rat. 1, 1302 and 1-19 cells.

line	Spl of pWTC1 (#4.0 DNA LCG)	T.C.	Incubation time
Rat. 1		21.1	5:00 pm
1302 -		17.0	5:45 "
1-19		16.9	6:40 "

RNA extracted according to protocol.

Cell no. for 1-19 cells was very low after electroporation, thus, needs to be repeated.

At 2nd isopropanol pptⁿ now

1-19 cell electroporation was repeated and samples replaced.

Digestion of probe pSTBB0.7 with EcoRI and gel analysis

Spl of pSTBB0.7 was digested with EcoRI for 3h in 50 µl R_x mix.

An aliquot of digested sample was analyzed on 0.6% agarose gel with undigested vector and the newly prepared pWTC1.

Thus:

Lane

- 1 - pSTBB0.7
- 2 - pSTBB0.7 ↓ EcoRI
- 3 & 4 - pWTC1

EcoRI Digest was heated to 65°C then ppt with 2.2 µl smol + 120 µl ethanol

-20° washed with 80% ethanol resuspended in TE at 1 µg/µl



RNA purification continued from page 179

20% (40 μ l) of each Rnt & lⁿ was coprecipitated with 20 μ g tRNA
and 5 μ l 3M NaAc pH 5.2 + 120 μ l ethanol, at -20°, ON.

Rest of Rnt samples were stored in presence of 500 μ l of ethanol at -70°C.

Probe was synthesized according to previous protocol, but
since my solutions were used up I used Benavito's solutions as
follows:

μ l		Actual
20.5	H ₂ O	
10.0	Sx Buffer *	
6.0	10 mM TTPS (3) *	
1.0	0.1 mM UTP *	
2.5	0.1 M DTT *	
1.0	RNA guard	
3.0	Template	2 μ l (2 μ g)
5.0	³² P UTP	7 μ l 3000 Ci/mmole
1.0	T7 polymerase	1.5 μ l
50 μ l		

Final concⁿ of cold UTP is 2 μ M instead of 12 μ M in my previous
experiment, and, of course, the molar quantity of hot UTP is 4 fold
lower. Therefore, in the present experiment must be at
least 10 fold NTP used.

RNA probe was dissolved in 1 μ l of hybridization buffer
2 μ l was used for counting = 162417

i.e. total CPM of probe = 500 \times 162417 = 8.1×10^7

Normally we use 1.6×10^7 CPM/ml (5×10^5 CPM/ 3μ l)
for hybridization. In the present situation it is
safe to use 3x more, thus using 1 part conc'd probe
to 2 part hybridization solⁿ will give 2.7×10^7 CPM/ml
1:1 is about 4×10^7 \rightarrow used.

For 7 samples need $15 \times 7 = 105 \mu$ l probe
+ 105μ l hybridⁿ solⁿ

✓ Spg tRNA used for control
Denatured at 85° / 5 min hybridized 48° o/n

normally we used 1.6×10^7 cpm /ml for hybridization
 5×10^5 /30 μ l

It is safe to use 3X more cpm than usual
: dilute the conc^d probe by 1:3

Sequencing gel with wedged spaces used 150 μ l "Kryol Sol"

RNA samples (20% of original) were dissolved in 12 μ l ^{Loading Buffer} 4 μ l (1/3) was used for gel loading.

- 1- Supplement nucleotide mixes with about 2X more CTP cold.
- 2- Cut DNA template by 1/2
- 3- add 3X more DNA marker.

Round II RNA Protection pWTC1

RNA samples precipitated with 10 μ l of TNA (100 μ g) + 22 μ l
3M NaAc pH 5.2 at -20°C / 30 min.

Spin at 12000 rpm 4°C. Redissolved pellet in 160 μ l DEPC H₂O.
40 μ l (20%) used for RNA protection, add with 9 μ l of 3M
NaAc pH 5.2 + 120 μ l of ethanol, -20 30 min. Spin 5 min 4°C.
redissolve each pellet in 30 μ l hybridization buffer + probe.

rNTP stock was made 250 μ M in CTP

2 μ l of 10 mM dNTP + 2 μ l H₂O, min, add 1.5 μ l to in 37 μ l of
old stock solⁿ. (rNTP - cistⁿ).

2 / 1000 μ l of purified probe tested = 240298 CPM

$$\text{total activity} = \frac{1000}{2} \times 2.4 \times 10^5 = 1.2 \times 10^8$$

Used 10 μ l of this probe in hybridization solⁿ + 20 μ l of
hybridization solution per sample. O/N incubation 45°C

RNAse 1, 30U (3 μ l), 75 min 37°C.

wedged gel small teeth used.

RNA samples dissolved in 12 μ l, 14 μ l (1 1/3) used
for gel loading, in 10% of Rat-1 + pWTC1 lost on loading.

Probe: 1 μ l added to 30 μ l of loading buffer mixed,
about 1.5 & 3 μ l loaded on gel. DNA markers diluted to
about 200 CPM / sample. Gel run at 1350 V

Loading order
 PBR 322
 DNA
 tRNA
 Rot-1 (-)
 Rot-1 (+)
 1302 (-)
 1302 (+)
 1-19 (-)
 1-19 (+)
 Probe
 DNA
 Probe

Xylene Cyanol FF comigrates with a RNA of about 110 nucleotides. Bromophenol blue with 25 nucleotides.

RNA has a lower mobility in these gels than DNA of the same size length. If DNA markers are used to estimate the size of protected ^{RNA} fragment, the correct size is 5 to 10% smaller than this estimate. For example if an Rnt species runs with a DNA marker of 100 nucleotides, its actual length is 90 to 95 nucleotides.

trial 2 Electroporation with pWTCL (new) 20 μ g / elect.

2 Rat-1 340 \checkmark Cap 260 μ F T.C. = 17.6

2 ethanol Incubⁿ at 11:40

LF2

4 1302 T.C. 16.9 Incubⁿ started at 12:35

C Both cell lines were split the day before
electroporation. trypsinization for 2 min

rd) After electroporation, cells were mixed inside a 15 ml tube with ~ 8 ml of medium, split to two plates, one plate to be used for DNA quantitation, the other for Rnt (approx. same no. of cells / plate).

DNA (nucleus) isolated 23h after and
Rnt 24h and 45 min after electroporation

119 Cells: electroporation began at 1:45 pm
T.C. = 17.3

λ phage

23.1

9.4

6.6

4.4

2.3

2.0

~~Cells were mixed~~

Rnt was purified according to standard protocol, finally dissolved in 50 μ l DEPC treated H₂O d-65/10 min. Further purification of Rnt samples were also carried out according to protocol. 20% of each Rnt sample was used for protection experiments.

New probe was synthesized with the remainder of DNA -

Probe Specific activity
 $\frac{2.1 \times 10^8 \text{ CPM}}{\text{total}}$

Dissolved in 1 ml of hybridization buffer

10 μ l (2.1×10^6) of this probe + 20 μ l of hybridization solution added to each ^{RNA} sample

Heated accidentally 7 min (instead of 5 min) at 85°, then incubated at 45°C for 16 h.

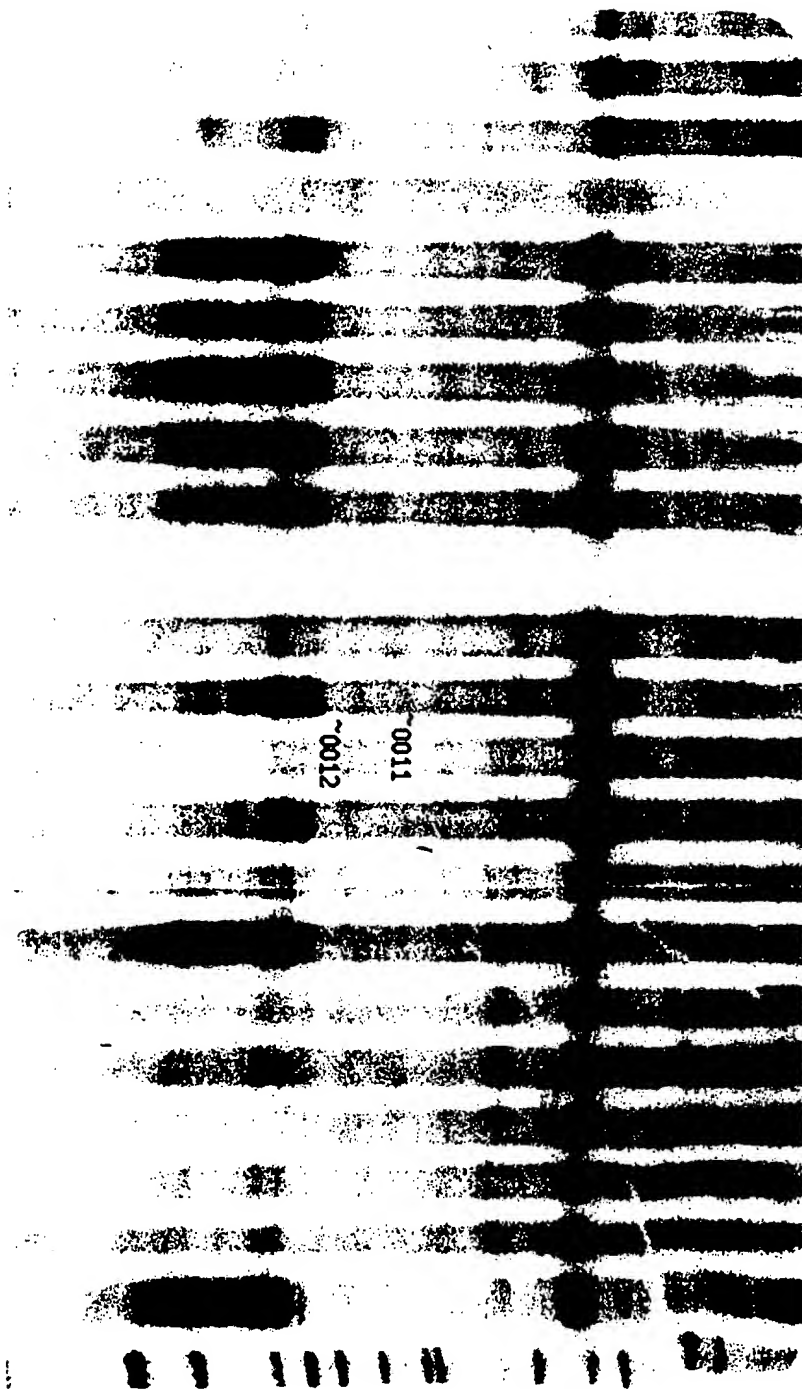
2 μ l (20 u) of Krase One added / sample digestion was for ~ 75 min.

Samples were finally resuspended in 15 μ l of loading buffer, 5 μ l was used for gel analysis.

2 vol of 1-19 Cells loaded on gel to match the cell numbers for all three cell lines, \therefore 2X DNA must be used

DATA\BB12.GED

17:13:01, Range = 4.99-100.00 Counts, 1.00x



Results of this experiment confirm the previous experiments results that:

- 1- The steady state level of ~~endogenous~~ endogenous Col $\alpha 1(I)$ mRNA in 1302 cells is at least 6 fold lower than in Rat-1 cells (compare C2 with C4). The steady state of the mRNA for 1-9 cells is intermediate between the two cell lines, i.e. about 3X higher than 1302 and 3X lower than Rat-1 cells.
- 2- Electroporation and/or transfection causes almost total degradation of endogenous $\alpha 1(I)$ mRNA and the level ^{of synthesis} does not return to normality up to 24h after electroporation (less than 10%). This observation is true for all three cell lines.
- 3- The exogenous $\alpha 1(I)$ gene does not seem to transcribe any mRNA up to 24h, i.e. it must be regulated the same way as the endogenous gene.
- 4- The ~~total~~ Col $\alpha 1(I)$ truncated promoter constructs synthesize mRNA efficiently within the 24h post-electroporation period. For example the human Col- hGH constructs with 800 bp 5' region, or the smaller mouse or rat Col- CAT constructs. Thus the phenomenon of lack of Col $\alpha 1(I)$ promoter expression within 24h is only relevant to the fully regulated gene.

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~~Electroporation of 1-15 with pW121 48h incubation~~

~~10 subconfluent plates~~

~~20µg DMT (10µg F1 + 10µg F2)~~

~~T.C. 15.0~~

~~Start electroporation at 2:00~~ ^{hourly}

CPT-3 control
positive control
- DMT

not

gate

11

not

gates

g

not

in 24h

Electroporation of Rat-1 (1302 and BMS-19 with
pWTC1 (20 ng) (100% F1 + 100% F2 CsCl prep) (48h)

Control cells: Same than cell lines
trypsinized 24h prior to harvesting with
electroporation, to differentiate between the
effect of electroporation and trypsinization
on Col. x(H) Rmt degradation and synthesis.

Rat-1 T.C. = 15.9 Incubation began at: 3:58

1302 15.5 4:50

BMS-19 15.8 5:50

Media and Rmt purifications were done
according to standard protocol.

Approximately equal no. of cells recovered on each
plate after electroporation.

Digestion of probe pST BB 0.7 with EcoRI

pST BB 0.7 DNA	1.45 µg/µl	5 µl (~ 7.5 µg)
10x R-React Buffer	3 µl	5 µl
EcoRI	2.2 µl	3 µl 60 u
H ₂ O		37 µl

120 µl
15000-75000
100%
100%
100%

37°C 3h → 65°C 10 min
ppt'd with 2.2 µl 5M NaCl + 120 µl 80% EtOH -20°C washed with 80% EtOH
resuspended in TE

h) γ - ^{32}P ATP phosphorylation of pBR322 LMBP

Fresh NEB DNA Sample	1 μl (1 μg)
H_2O	20 μl
(no heating)	
10x Kinase buffer NEB	3 μl
γ - ^{32}P ATP Fresh	5 μl
T4 polynucleotide kinase	1 μl
	<u>30 μl</u>

50 min at 37°C

After spin column purification, 40% of label was incorporated. 60% was left on the column.

Probe was synthesized according to protocol. After column purification was resuspended in 1 μl of hybridization buffer. 10 μl of this solution + 20 μl of cold hybridization buffer added to each RST sample (1/5 of each RST sample used).

3 μl of probe solution was taken for scintillation counting which gave 1.3×10^4 counts.

Thus CPM/sample is 13×10^5 in 3 μl .

Results of this experiment were almost identical to 24h postelectroporation, i.e. all (1) Collagen mRNA synthesis has not resumed up to 48h.

PCR

10 X Buffer	10 μ l	X 12
Primer Lac1 / Lac2 Mix	20	120 ✓
dNTP mix	1.4	240 ✓
α 32 P dCTP (5 weeks old)	3	16.8 ✓
DNATag polymerase	0.4	36
the	55	4.8
	89.8	660 ✓

DNA

10 μ l

File #32

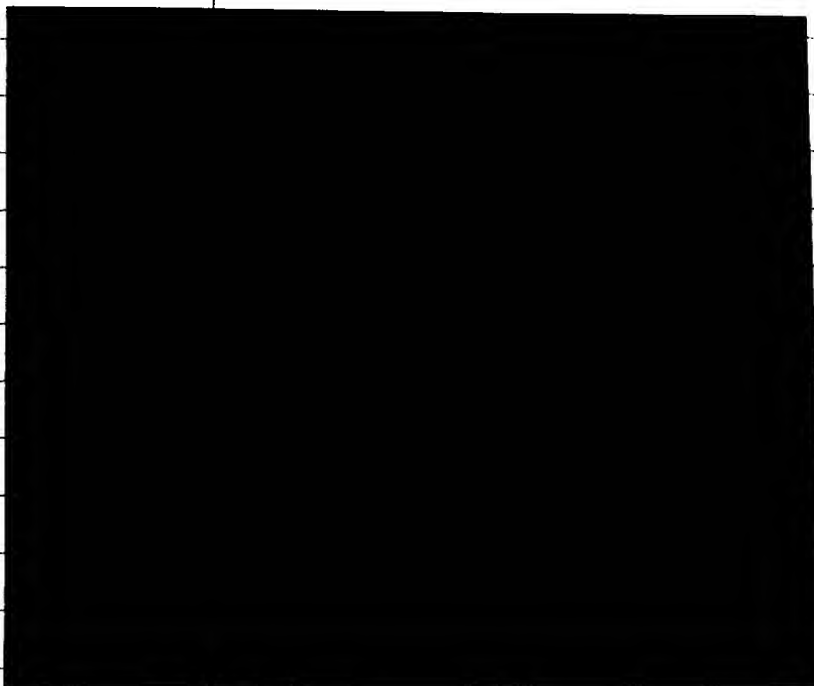
10 cycles

1", 94°; 2", 53°; 2", 70°

DNA standards PBAG 50, 10, 5, 1 and 0.1 μ gLac primers did not work on pUTC1 templates, probably because Lac gene is deleted.
(insert cloned in pBR322)

New primers, AmpS and Amp3 were ordered.

Lanes 1-5: PBAG; 6: Control DNA; 7-12: Rd1, 1302 at 1-19 cells 24 and 48h post electroporation

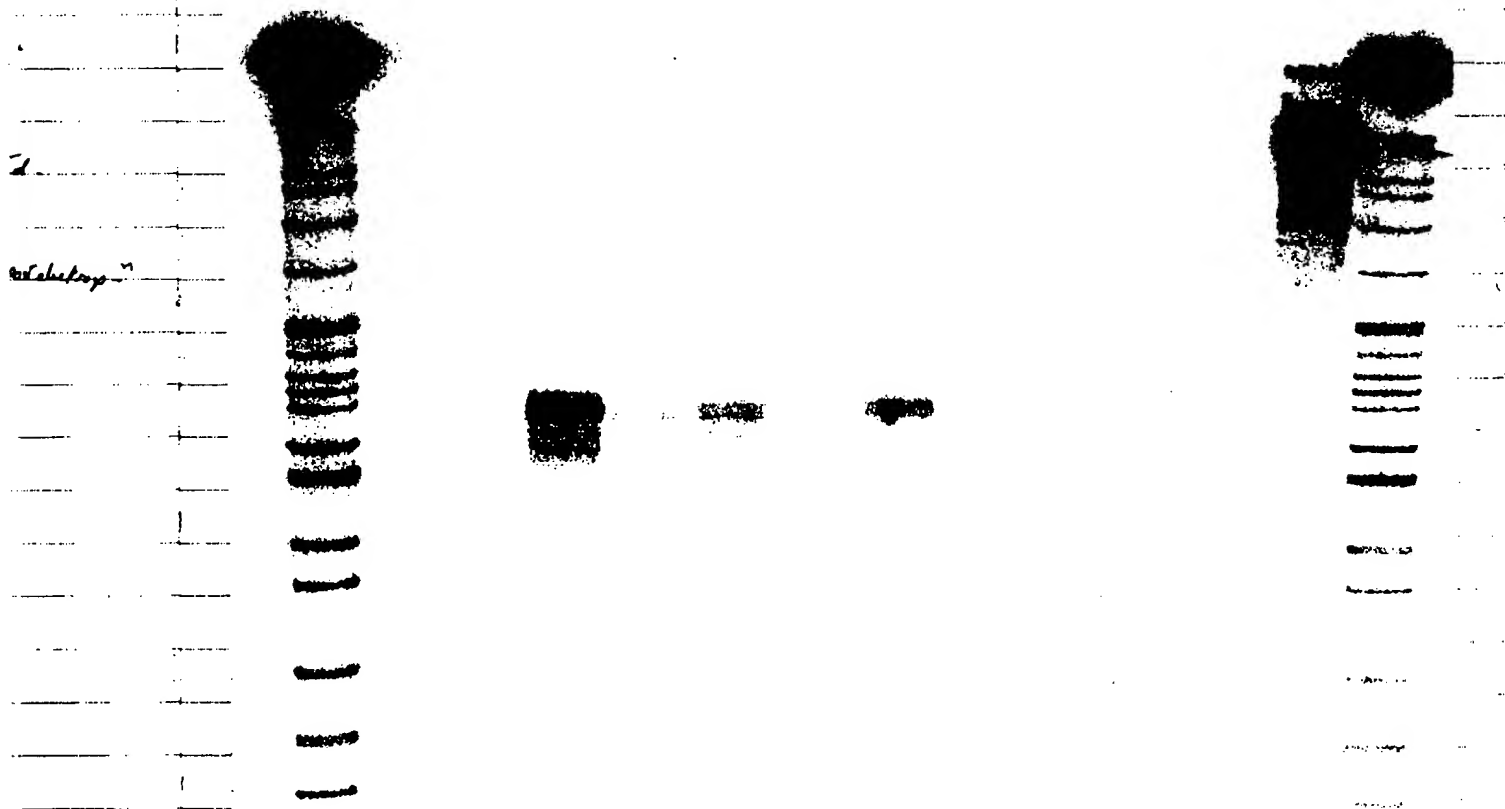


203

3-1870

A\PWTC48.GEL

12:26:25, Range = 9.98-199.88 Counts, 1.00x



~~The~~ ~~transfection~~ of pWTC1 into Rat1 /1302 and 1-19 cells by DEAE-Dextran-extended protocol.

This was done with 10 μ g of DNA in each case according to protocol on page 203.

Cell survival in all three cell lines were very poor, less than 5%. An entire ~~electroporation~~ ^{transfection} of 1-19 cells was done without DNA. The yield of cells was just as poor.

went through with Rnase and Rnase prep after 48h incubation. 10 μ g of Rnase was added to each sample when Guanidine thiocyanate was added, to help ~~carry~~ carry the Rnase through the procedure. intend to use 1/2 of each Rnase + DNA sample for each determination. \rightarrow assuming about equal no. cells recovered per plate.

These Rnase samples will be analysed ^{along} with unelectroporated Rat1, 1302 and 1-19 Rnase using another probe such as GAPDH as internal control.

Amp Primers for estimation of Transfected pWTC-1

The cosmid pWTC-1 carries the Amp gene.
Two primers were ordered to amplify a 223 bp fragment of Amp^r gene. They are designated Amp-S and Amp-3 sequences are attached.

Amp-S was delivered at 13.1 A₂₆₀ 48 µg / A₂₆₀ u
= 628.8 µg

$$\begin{aligned} 65 \mu\text{g in } 100 \mu\text{l} &\rightarrow 100 \mu\text{M sol}^n \\ 628.8 \mu\text{g} &\div \frac{100 \times 628.8}{65} \end{aligned}$$

$$= \underline{967 \mu\text{l}} \quad 100 \mu\text{M sol}^n$$

Amp-3 11.1 A₂₆₀ at 47 µg / A₂₆₀
= 521.7 µg

$$\div \frac{100 \times 521.7}{65} = \underline{803 \mu\text{l}} = 100 \mu\text{M}$$

Mix Amp-S with Amp-3 at equal vol to
give 50 µM solution of 2 oligos.
Can use 2-4 µl / reaction.

Testing The Amp Primers

The amp primers Amp-5 and Amp-3 mentioned were tested in Cetus PCR Assays as follows:

- #1) 1 μ l of E3 assay tube pWTC1 Dmt
 #2) 5 μ l of 10 ng/ μ l pBAC Standard (we don't know if pBAC has the Amp^r gene)

	1 sample	2 sample
10 X Buffer	10	20 μ l
Amp primers	4	8 μ l
dNTP	1.5	3
tag polymerase	0.5	1
H ₂ O	82	164

File #32 25 cycles

After PCR the products (10 μ l each) were analyzed on 1% agarose gel along with ϕ X174 Hae III markers and two plasmids recovered from Berritt: pGroot and pLS-1, for quantitation. 1/2 μ l of the two plasmids loaded on gel.

Thus:

Lane 1 = ϕ X174 Hae III

Lane 2 = pWTC1 PCR products \rightarrow pure plasmid

3 = pBAC + rat genome Dmt

4 = pGroot

5 = pLS-1



Conclusions: Primers work well on both pWTC1 and pBAC templates. At least two additional bands apparent in the gel come from genomic Dmt. pGroot concⁿ is ~ 1 μ g/ μ l pLS-1 ~ 3-4 μ g/ μ l. ✓

Further Studies on The Fate of Endogenous Collagen $\alpha 1(I)$ mRNA after electroporation

There are a number of questions to be asked regarding the mechanism of Collagen $\alpha 1(I)$ mRNA degradation and cessation of synthesis:

- 1- Is it electroporation per se responsible of the shut off or electroporation + DAA?
- 2- Is it the cosmid with the complete $\alpha 1(I)$ gene which is titrating cellular transcription factor(s) and resulting with the lack of endogenous mRNA?
- 3- Does the synthesis of $\alpha 1(I)$ mRNA resume after several days, or the process is irreversible?

In order to answer the first question on electroporation of Kd-1 cells was done without DAA, incubation was continuous for under 48 h initially, some of the cells were removed for mRNA analysis, the rest repeated and continued incubation for over a week to take care of questions 1 and then 3.

The answer to the second question is already partly answered. Electroporation with cosmid does not result in cosmid expression, neither the cosmid nor the endogenous gene are expressed.

Our own electroporation is never 100% efficient, thus it should not result in 90-95% drop in the level of endogenous gene expression.

In any case, an electroporation was done with 3 μ g of pCol CAT-3 which carries the basal $\alpha 1(I)$ promoter construct. It is electroporated very efficiently, we also know that it expresses CAT efficiently, we shall investigate if it with turn off endogenous $\alpha 1(I)$ mRNA.

Cells were harvested, for CAT-3 / Rat-1 at 45 h, plate was 80% confluent, compared to cosmid electⁿ which was about 6-7 conflⁿ. 1/2 cells to RNA prep, the other 1/2 to DAA.

Electroporated cells without plasmid were 60% confluent at 48h. 40% of the cells were used for last assays, the rest of the cells were split on diff plates for further incubation and study.

So about 1/4 of each last sample should be equivalent to previously electroporated cells, but again internal control needs to be used to be able to compare results.

Electroporated cells harvested again at 5 days & 7 days post-electroporation. At 5 days a 30% confluent plate used, at 7 days plate was 90% confluent. Thus Rest conc's need to be determined before protection experiments.

PCR of all DMT samples with New AMP primers

	1x	17x
10 x Buffer	10 μ l	170 \checkmark
Primers Amp 3 / 5	4	68 \checkmark
dNTP mix	1.4	24 \checkmark
α γ p dCTP	1	17
Taq polymerase	0.4	7
H ₂ O	73	1241 \checkmark
	<u>90 μl</u>	
	10 μ l	

DMT

File #32 / 33 12 cycles.

Lane 1 = Rat-1 / pMT1, 24h post electroporation

2 = 1302 " " " "

3 = 1-19 " " " "

4 = Rat-1 / pMT1, 48h post electroporation

5 = 1302 " " " "

6 = 1-19 " " " "

7 = Rat-1 / pMT1 48h post DEAE-DEX Transfection

8 = 1302 " " " "

9 = 1-19 " " " "

10 = Rat-1 genomic DNA

11 = genomic DNA + pBAG 0.1 ng / 10 μ l

12 = " " " " 1 " "

13 = " " " " 5 " "

14 = " " " " 10 " "

15 = " " " " 50 " "

16 = Rat-1 / pCD13, 45h post electroporation - 10% DNA used

* 10% of all PCR reactions analyzed on gel.

10% of
DNA used

50% of
DNA used

Results show that all transfection experiments worked equally well. Controls did not work, presumably pBMT does not have the Amp gene. Control genomic not crosscontaminated with adjacent samples.

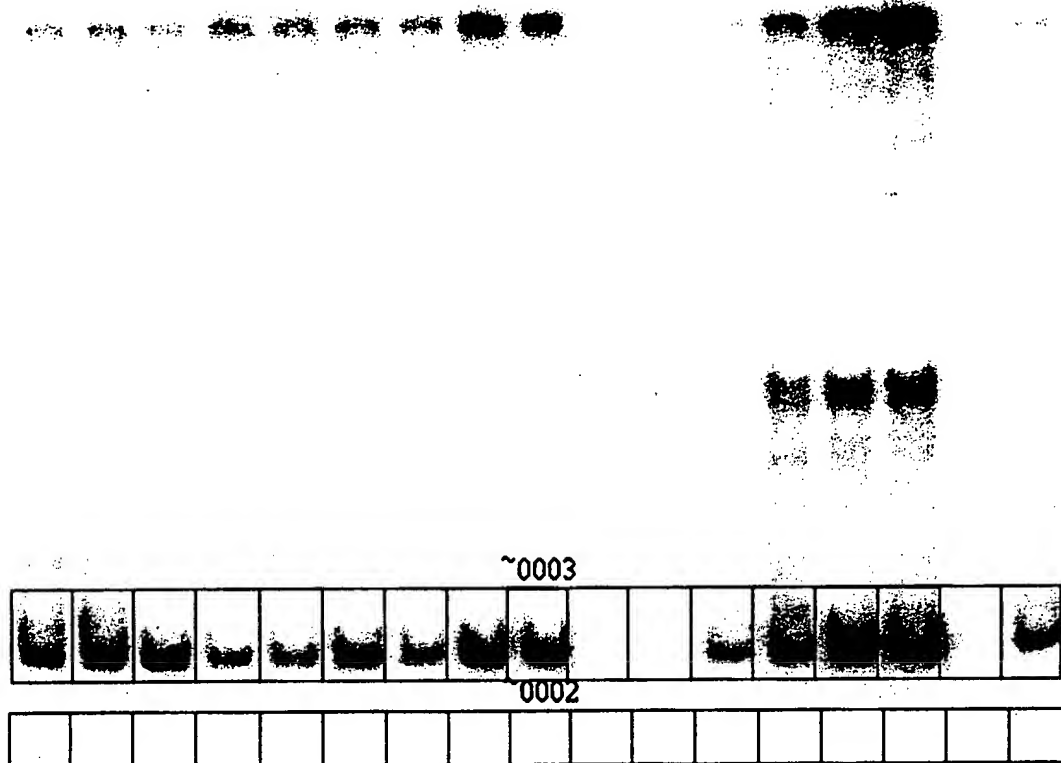
PCR was repeated for the ^{new} controls under exactly the same conditions as the last experiment.

The new standards are made from pCol CAT 3. Concentrations are: 1, 10, 50 and 100 ng / 10 μ l. Negative control, genomic DNA was also repeated. 5 μ l of each reaction loaded on the gel.

1 space allowed on gel between samples 9 and 10, and between 100 ng standard and pCol CAT 3 / RAT 1 DEX. Gel run at 200 V

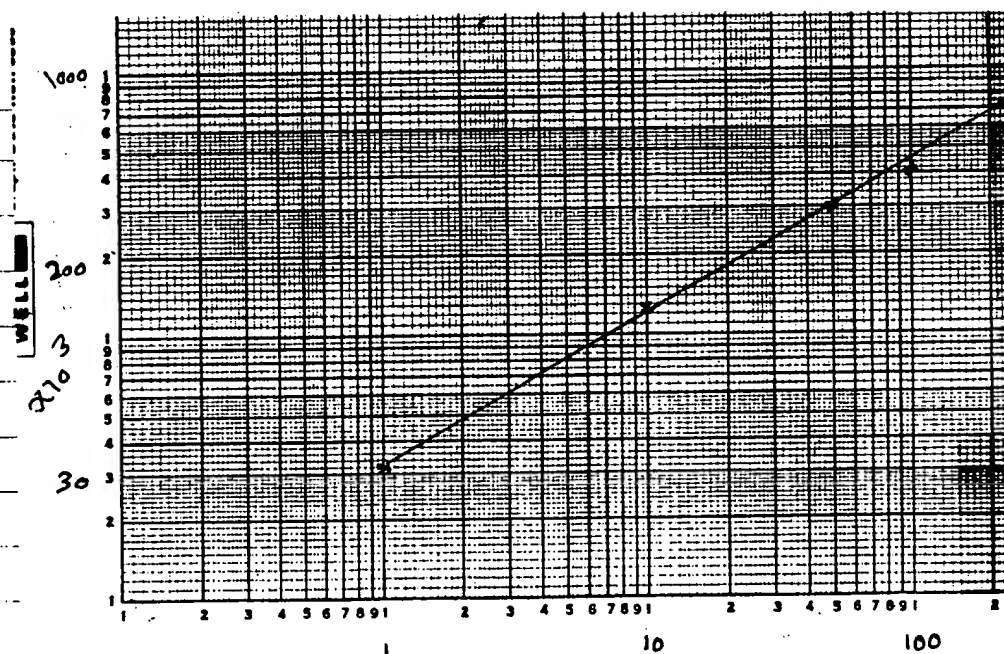
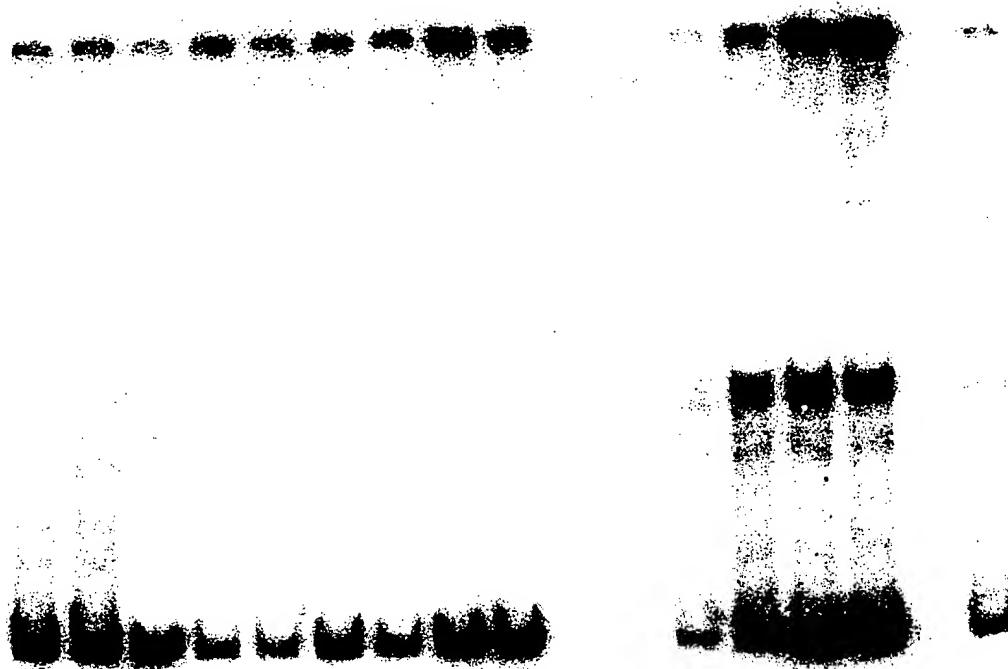
3 μ l of BBP dye + 5 μ l sample -
Gel #2: ^{about} 20% of the sample in Lane #2 was accidentally lost - floated.

The second gel worked fine all of the standards showed up with O/N exposure and Genomic DNA did not.



OBJECT NAME	VOLUME	ng	BACKGROUND	# PTS IN OBJECT	SUM ABOVE BKGND	Copy #
*0003-R1C1	135099	12	11.65	3243	136823	24 x 10 ⁹
*0003-R1C2	133605	12	14.18	3243	135889	30 x 10 ⁹
*0003-R1C3	133183	12	11.06	3243	138977	24 x 10 ⁹
*0003-R1C4	29565	0.92	8.836	3312	33412	1.84 x 10 ⁹
*0003-R1C5	32578	1	8.423	3243	35498	2 x 10 ⁹
*0003-R1C6	77621	4.6	8.065	3243	81280	9.2 x 10 ⁹
*0003-R1C7	40320	1.4	9.031	3312	45322	0.56 x 10 ⁹
*0003-R1C8	142898	13	14.03	3243	150019	5.2 x 10 ⁹
*0003-R1C9	124376	10.5	19.63	3243	138473	4.2 x 10 ⁹
*0003-R1C10	-15962		11.63	3243	4899	
*0003-R1C11	-376.2		3.427	3312	1286	
*0003-R1C12	32414		7.658	3243	34399	
*0003-R1C13	123946		17.12	3243	128648	
*0003-R1C14	305291		27.42	3312	315661	
*0003-R1C15	405947		30.79	3243	417063	
*0003-R1C16	-18125		15.00	3243	6108	
*0003-R1C17	50548	2.2	6.263	3243	62442	4.4 x 10 ⁹

Gene Copy # = ng (madamgraph x 2 x 10⁸) x 1 / Fraction of DNA used in PCR



Amp
 Gene
 Copy No
 8.4×10^3
 3.0×10^3
 2.4×10^3
 1.8×10^3
 2×10^3
 9.2×10^3
 0.56×10^3
 5.2×10^3
 4.2×10^3

4.4×10^3

10×10^8 Copy No (Pre-CAT. 3)

Digestion of probes pLS-1 and pG-ract with Hind III

pLS-1 (1 μ g/ μ l)	10 μ l	
BRL 10X React Buffer 2	5 μ l	
Hind III 20u/ μ l	2 μ l	
H ₂ O	33	
	50 μ l	
pG-ract 1 μ g/ μ l	2.5 μ l	
BRL 10X React Buffer 2	5 μ l	
Hind III 20u/ μ l	2 μ l	
H ₂ O	40 μ l	
	50 μ l	37°C / O/N

Analysis of RNT

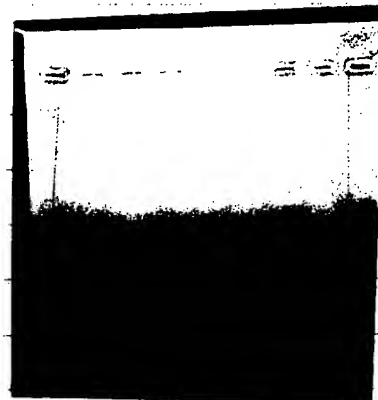
RNT Samples from Rat 1 electroporated after 2, 5 and 7 days and CAT-3 / Rat-1 elect. 48h, and unelectroporated Rat-1 (48h) were quantitated. 5% of each Rat sample ~~(total) and sent for 7 Days 2.5% were sent with~~
~~RNT then stored, digested and analyzed on polyacrylamide gel~~

Analyzed on 1.5% agarose gel along with Hind III digested probes: (1 μ l of 20 μ l solⁿ in TE)

Lane 1: pGract
 Lane 2: PLS-1
 Hind III

Lane

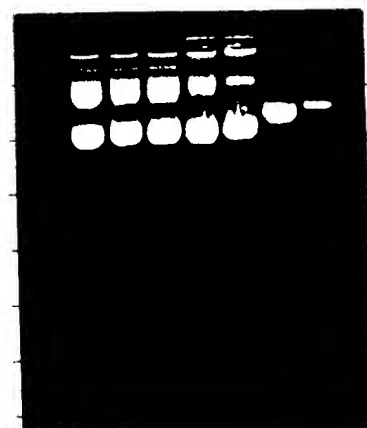
- 4% 13: unelectroporated Rat-1 RNA (48h)
 16% 24: Elect'd Rat-1 RNA, 45h
 30% 35: " " " 5 days
 30% 2.5% used ← 48: " " " 7 days
 40% 57: Rat-1 electroporated with pGract-3, 45h
 6) PLS-1 / Hind III ↓
 7) pGract / Hind III ↓



PLS-1 seems at least 2X stronger than pGract; ∴ PLS-1 was diluted 2X so that both probes will be about 0.5 µg/µl

The RNA concentrations do not seem to correspond to visual cell no. Could also be artifact since electroporation was done in TBE pH 8.2 while RNA may not be so stable.

Experiment was ∴ repeated.



<u>cell/ Electroporation</u>	<u>visual confluency</u>	<u>Actual cell no. Coulter counter</u>	<u>Resuspend RMT in x μl TE</u>
<u>Summary</u>			
unelectroporated	60-70%	1,840,000	184
Elect <u>2 Days</u>	90%	3,200,000	320
" <u>4 Days</u>	80%	2,040,000	204
" <u>6 Days</u>	70%	2,080,000	208
Electroporated Rat/CAT-3 - 2 Day	80% \rightarrow 1/2 to RMT, \approx 40%		102

Use 20 μ l of each RMT solⁿ / experiment

+ 2 μ l tRMT

+ 2.5 μ l 3M NaAcetate pH 5.2

+ 75 μ l ethanol

\rightarrow -20°C

3.0 min

Spin 4°C / 5 min



Repeat of Electroporations of Rat-1, different cell harvest time,
 counting of cells by Coulter Counter.

Un-electroporated Rat-1 cells look about 60-70% confluent

Electroporated Rat-1 cells after 2 days look about 90% confluent

Cell counts: Elect 2 Days 8000
 Un-electroporated 4600

4645

$$8000 \times 40 \times 10 = \underline{3,200,000} \quad - 21)$$

$$4600 \times 40 \times 10 = \underline{1,840,000} \quad - \text{un-electroporated}$$

4 Day cells look about 80% confluent:

$$5100 \times 40 \times 10 = \underline{2,040,000}$$

5 Day cells look ~ 70% confluent

$$5200 \times 40 \times 10 = \underline{2,080,000}$$

Sequencing gel:

2/20/ 50 ml 6% acrylamide / 1 x TBE / 7.7 M urea
 400 µl 10% APS
 4 µl TBE.

Lane: 1) pB2322 ↓ Msp I

Exp 1: Electroporation of Rat-1 / B02 / 1-19 with pWTC1 - 24h post electⁿ

2) Rat-1, unelectroporated

3) Rat-1 Electroporated

4) B02, unelectroporated

5) B02 electroporated

6) 1-19, unelectroporated

7) 1-19 electroporated

Exp 2: Electroporation of Rat-1 / B02 / 1-19 with pWTC1 - 48h post electⁿ

8) Rat-1 unelectroporated pretransfected (P/T)

9) Rat-1 Electroporated

10) B02 unelectroporated P/T

11) B02 Electroporated.

12) 1-19 unelectroporated P/T

13) 1-19 Electroporated

Exp 3: Electroporation of Rat-1 cells with pCol CAT-3 48h

Exp 4: 14) Rat-1 / pCol CAT-3 48h post electroporation

Exp 4: Unelectroporated cells vs Electroporated Rat-1 cells, 2, 4 and 6 days post electⁿ

15) Rat-1 unelectroporated

16) Rat-1 Electroporated no plasmid 2 days post electroporation

17) " " " " 4 " " "

18) " " " " 6 " " "

19) 2 Rat-1 Control

Expt 5: Transfection of pWTC1 into Rat1, 1302 and 1-19, DEAE-DEX 48h

20) Rat1 /pWTC1 DEX 48h

21) 1302 /pWTC1 DEX 48h

22) 1-19 /pWTC1 DEX 48h

23) 1-19 ~~unelectroporated~~ DEAE-DEX treated

24) Mixed 3 probes in the ^{proportions} ~~ratio~~ added to hybridization mixture

25) probe pST BB0.7

26) probe pLS-1

27) probe pGratt - negative control for RNA protection

28) pBR 322 \downarrow MspI

	24 h post electroporation										48 h
	Rat-1 unelectroporated	Rat-1 + pWTC1	1302 unelectroporated	1302 + pWTC1	1-19 unelectroporated	1-19 + pWTC1	Rat-1 unelut. + hypoxanthine	Rat-1 + pWTC1	1302 unelut. + hypoxanthine	1302 + pWTC1	1-19 unelut. + hypoxanthine
$\alpha 1(I)$ (main bands, 2)	48727	7128	6007	1942	9094	6184	85264	11223	26820	5357	48793
- 1/2 tRNA (280)	45927	4328	3207	6294	3384	82464	8423	24020	2557	45543	
							128,000				
GAPDH	45902	28635	98658	91673	43298	122744					
- 1/2 tRNA (280)	44951	43730	25752	36590	27847	39501	11391	38684	17380	65275	15437
$\alpha 1(I) / GAPDH$	1.023	0.000	0.124	0 ^{up}	0.226	0.086	0.724	0.218	0.138	0.039	0.29
							1.05				

 $\alpha 1$

GAPDH

OBJECT NAME VOLUME

"0005-R1C1 2.50 4780

"0005-R1C2 2.54 8118

"0005-R1C3 0.72 2582

"0005-R1C4 6.08 11417

tRNA "0005-R1C5 - 2807

OBJECT NAME VOLUME

"0002-R1C1 18677

"0002-R1C2 12285

"0002-R1C3 35873

"0002-R1C4 18776

tRNA "0002-R1C5 3830 1

Oligos Etc.

P.O. Box: 874

Ridgefield, CT 06877

Rule 131-Petition -- AN 09/472,558

FAX # 800-869-0813 Page 34 of 48

Phone: 800-888-2358

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Phone: (617) 253-7666

BILLING ADDRESS: MIT, P.O. Box 69, CAMBRIDGE, MA 02139

Oligo # AMP-5:

5' GTA GTT CGC CAG TTA ATA GT 3' 20-mer

Small scale

Oligo # AMP-3:

5' GCT GCC ATA ACC ATG AGT GA 3' 20-mer

small scale

Date required by: ASAP

40 nucleotides at \$4.50 = \$180.00

Feb. 23-1995

M.B. Bahramian

Amplifies a 223 bp fragment from Ampicillinase gene cloned into pBR327

Sober, X., Covarrubias, L. and Bolivar, F. (1980) construction and characterization of new cloning vehicles. IV. Deletion derivatives of pBR322 and pBR328 - Gene 9, 287-305.

48h Post-transf.

48h posttransfection

1020 5357 48393 11193 46214 62539 63824 37708
 1020 2557 45543 8393 43414 59750 61084 34908
 1301 65275 154372 57075 88930 59500 53751 44382
 2.139 0.039 0.295 0.147 0.488 1.004 1.135 0.787

57216 10284 1449 1883 3617
 54416 7404 10017 1182 1716 3330
 81330 14946 92043 8195 14247
 63163 16776 33873 10265 16677
 0.262 0.597 0.035 0.167 0.201
 0.250 0.548 0.045 0.225 0.244

 $\alpha(1)$

GAPDH

OBJECT NAME VOLUME
 L-RNA
 "0014-R1C1 5585
 Rat1 6D "0014-R1C2 57218
 Rat1 4D "0014-R1C3 37708
 Rat1 2D "0014-R1C4 63824
 Rat1 1A "0014-R1C5 62539
 Rat1/CAT3 "0014-R1C6 48214
 "0014-R1C7 6857
 Blank
 1-19/pwrc1 "0014-R1C8 11193
 1-19 "0014-R1C9 48343
 1302/pwrc1 "0014-R1C10 5357
 1302 "0014-R1C11 26820
 Rat1/pwrc1 "0014-R1C12 11223
 Rat1 "0014-R1C13 85284
 1-19/pwrc1 "0014-R1C14 6184
 1-19 "0014-R1C15 9094
 1302/pwrc1 "0014-R1C16 1942
 1302 "0014-R1C17 6897
 Rat1/pwrc1 "0014-R1C18 7138
 Rat1 "0014-R1C19 48727

OBJECT NAME VOLUME
 GAPDH
 "0008-R1C1 4089
 "0008-R1C2 65163
 "0008-R1C3 46382
 "0008-R1C4 55751
 "0008-R1C5 61500
 "0008-R1C6 90830
 "0008-R1C7 1734
 "0008-R1C8 59075
 "0008-R1C9 156372
 "0008-R1C10 68275
 "0008-R1C11 175901
 "0008-R1C12 40684
 "0008-R1C13 115911
 "0008-R1C14 41501
 "0008-R1C15 29847
 "0008-R1C16 38590
 "0008-R1C17 27752
 "0008-R1C18 45730
 "0008-R1C19 46951

15:55:02, Range = 1.00-299.89 Counts, 1.00x

DATA\PWTC-22.GEL

Blank

Rect 1

Rect 1 + PWTC1

1302

1302 + PWTC1

119

119 + PWTC1

OBJECT NAME	VOLUME	BACKGROUND	# PTS IN OBJECT	SUM ABOVE 100000
"0008-R1C1	24027	27.17	2485	8888
"0008-R1C2	208881	78.54	2485	28888
"0008-R1C3	15484	24.88	2485	16788
"0008-R1C4	21510	19.31	2485	28888
"0008-R1C5	2370	17.88	2485	4888
"0008-R1C6	58554	47.21	2485	88788
"0008-R1C7	13681	25.94	2485	18884

Electroporation of Rat-1, 1302, 1-19 Cells with:
CAT-1 (3.6 kb), CAT-2 (2.5), and CAT pVIT (1.1 kb) and
CAT-3 (0.22 kb).

Determination of transcription and translation in vivo
RNA / Cell extract and Nuclei isolations (each
electroporation divided 3 ways).

Electroporation of NIH 3T3 Cells with pVITC1

RNA / DNA isolation - determine that rat & mouse cells
behave similarly with respect to $\alpha(1)$ shut off
by pVITC1.

Not enough CAT-1 was present, so it was
prepared by boiling method. The yield of plasmid
was bad, so some old and some new plasmid
were mixed and used in various electroporations.

Viability of 1-19 cells after electⁿ was good
1302 & Rat-1 cells not so good (about 1/4 to 1/5
of 1-19). 3T3 cells recovery was bad.

3T3 cells were re-electroporated same day
the second prep was OK, same losses
recovery as 1302 & Rat-1.

Following experiments are planned to eventually run on 4 gels

- ① 4 different $\alpha 1$ (I) promoter - CAT gene constructs transfected into Rat-1, 1302 and NIH 3T3 cells (12 samples) + 3 controls Rat-1, 1302, NIH 3T3 untransfected (48 h after electroporation) + 1 tRNA lane (16 samples + DNA and protein lanes)

Mix CAT probe + GAPDH probe (only to assess positive control for hybridization and to show that comparable number of cells were used DNA det^d by PCR will show the relative rates of transcription.

- ② Exactly the same Rat samples as in ① used but probed with $\alpha 1$ (I) probe and GAPDH probe to investigate the extents of suppression of endogenous rat $\alpha 1$ (I) collagen gene by different lengths of their transgene promoters

- ③ Effect of exon-intron region and whole gene pWTC1 on Rat-1, 1302 and NIH 3T3:
- | Rat-1 Rat Control | Rat-1/pST-882-6 | Rat-1/pST-880-7 | 1302 Control | 1302/pST-882-6 | 3T3 Control | 3T3/pST-882-6 | 3T3/pST-880-7 | 3T3/pWTC1 |
|--------------------------|-----------------|-----------------|--------------|----------------|-------------|---------------|---------------|-----------|
| + tRNA lane (10 samples) | | | | | | | | |

Probe with $\alpha 1$ (I) (pST880-7 \downarrow EcoRI antinuc) and by GAPDH \times Endogenous and exogenous $\alpha 1$ (I) protected fragments will comigrate, except in the case of pWTC1 transfected 3T3 cells. The short promoter + exon-intron regions ^{are expected to improve $\alpha 1$ (I) mRNA} should theoretically lead to ~~an~~ increase in the total $\alpha 1$ (I) mRNA / GAPDH mRNA ratio. If the opposite takes place, then murine / rodent promoters and human $\alpha 1$ (I) gene are not regulated by the same factors.

Electroporation of pSTBB 0.7 and pSTBB 2.6 into NIH-3T3, Rat-1 cells and 1302.

The objects of this experiment is to verify if the shorter or longer intronic/exonic sequences have any effect on expression of the endogenous $\alpha(1)$ in both Rat-1 cells and in NIH 3T3 cells. Also want to investigate if the short mouse promoter with the entire 5' intron/exon sequence ^(pSTBB 0.7) is expressed to similar levels in 1302 cells.

Thus we electroporated Rat-1 & 3T3 cells with 0.7 or 2.6 plasmids at 1302 with just 2.6 plasmid, a control NIH3T3 was also electroporated at the same time with no DNA for verification of endogenous $\alpha(1)$ mRNA control.

RNA probes to be used are pSTBB 0.7 and GAPDH also RNA will be determined.

We could also use ~~antiserum~~ ^{antiserum} pSTBB 0.7 for antiserum mRNA synthesis in ratyubin.

Recovery of cells after electroporation was good for Rat-1 & 1302 cells but not good in NIH-3T3 cells (30%), with only a 1.5% for Rat-1 about 90% after 48h.

So for Rat-1 and 1302 we should use 1/5 of Rest for 3T3 1/2 of Rest.

$\alpha 1(1)$ sense RNA probe pST BBO.7 was
digested with PST-1 ^{NEB, EcoRV, buffer #3} 4h, heated to 65°C / 15 min
10 μ g in 20 μ l react. vol.
without further purification proceeded to in vitro
synthesis.

PBR322 \downarrow MspI was labelled with γ 32 P
to about 10⁶ cpm / 2 μ g DNA with about
60% of label incorporated into DNA on spin column
purification. 2 μ l of 300 μ l eluate was mixed
with 50 μ l of RnT loading buffer which gave
100,000 cpm / 50 μ l
2,000 cpm / μ l less than a μ l suff. and on gel

3 probes (pST BBO.7, $\alpha 1(1)$); pLS-1, GAPDH; and
pTRI-CAT) were transcribed by T7 RnT polymerase
according to protocol.

A second part of this investigation is to verify that short promoter + the enhancer elements is ~~to be~~ 2-fold less efficiently expressed in 1302 cells compared to Rat-1, in accordance with the data on human procolagen $\alpha 1(I)$ - hGH reported results.

\therefore Data data needed

(4) To investigate if significant increase in anti sense $\alpha 1(I)$ mRNA results in shut-off of this gene.

24h posttransfection

Rat-1 (Control)	Rat-1/ pWTC1	1302 pWTC1	1-10 pWTC1	3T3 pWTC1
--------------------	-----------------	---------------	---------------	--------------

Rat-1/ BB0.7	Rat-1/ BB2.6	1302/ BB2.6	3T3/ 0.7	3T3/ 2.6	tRNA
-----------------	-----------------	----------------	-------------	-------------	------

Probe with sense $\alpha 1(I)$, BB0.7 \downarrow PST-1
+ GAPDH

4 probes to be synthesized: BB0.7 sense + anti sense
GAPDH and CAT

1/3 of the above RNA samples to be used / experiment

except for 24h elut. Rat-1 1302 1-10 any have in the remaining rows

Results:

1st gel (CAT / GAPDH) came out black in all lanes including control
presumably because RNT contained some NaOH which might inhibit
reaction. Thus, this experiment should be repeated with 1/3 of
RNT samples left, with new probes.

Gel #2 came out ok. ($\alpha 1(1)$ / GAPDH)

		$\alpha 1(1)$ GAPDH		$\alpha 1(1)$ GAPDH	
tRNA	OBJECT NAME	VOLUME	VOLUME		
Rat-1	"0013-R1C1	10552	1083	1.56	1.02
Rat-1 3.6 Kb	"0013-R1C2	84058	53787	0.74	0.47
" 2.5	"0013-R1C3	53236	71792	0.68	0.44
" 1.0	"0013-R1C4	131513	183460	0.75	0.48
" 0.22	"0013-R1C5	212068	283413	0.77	0.49
1302	"0013-R1C6	162131	235120	0.45	0.16
1302 / 3.6 Kb	"0013-R1C7	22918	50539	0.27	0.05
" 2.5	"0013-R1C8	5051	18614	0.18	0.05
" 1.0	"0013-R1C9	18825	104257	0.18	0.05
" 0.22	"0013-R1C10	31147	174568	0.14	0.05
1-19	"0013-R1C11	28895	206870	0.49	0.25
1-19 / 3.6	"0013-R1C12	24611	50129	0.52	0.26
" 2.5	"0013-R1C13	49208	94022	0.39	0.20
" 1.0	"0013-R1C14	38977	100697	0.35	0.18
" 0.22	"0013-R1C15	45738	125228	0.43	0.22
	"0013-R1C16	38801	91751		



Gel #3 : 1/2 of the samples related to Rat cells came out ok.
 but mouse cells did not work since mouse and rat
 GAPDH are different and specific mouse protein must be used

	$\alpha 1(1)$	GAPDH	$\alpha 1(1)$ GAPDH	
RNA	OBJECT NAME	VOLUME	VOLUME	
Rat-1	"0020-R1C1	111.4	4844	1.19
Rat-1	"0020-R1C2	85645	72040	0.36
Rat-1/B82.6	"0020-R1C3	148279	407730	0.44
Rat-1/B80.7	"0020-R1C4	87764	198905	0.27
B302	"0020-R1C5	29351	107966	0.14
B302/B82.6	"0020-R1C6	40784	280830	0.03

$\alpha 1(1)$
 GAPDH(rat),
 $\alpha 1(1)$ / GAPDH



2K.

W-sensel

	OBJECT NAME	VOLUME	BACKGROUND	# PTS IN OBJECT	AVERAGE
$\alpha 1$	3T3 01	26712	17.99	1581	34.68
	3T3/26 02	9628	15.28	969	25.22
	3T3/07 03	12067	18.73	1007	28.72
	3T3/pw1c1 04	1502	13.86	429	17.16
GAPDH	3T3 05	27211	18.63	1035	44.92
	3T3/26 06	26215	17.43	1225	38.68
	3T3/07 07	50919	18.89	1595	50.91
	3T3/pw1c1 08	5225	12.57	1025	17.68

3-3 0.98

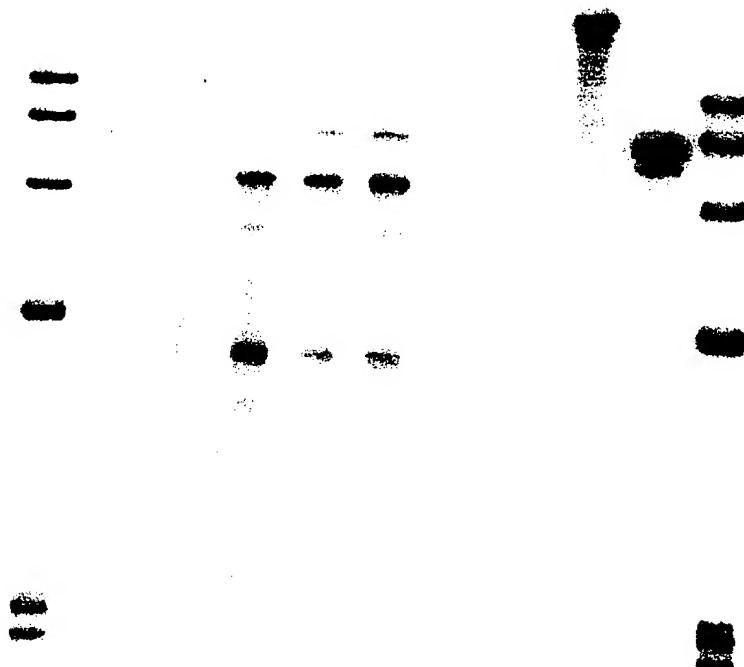
3T3/26 0.36

3T3/07 0.24

3T3/pw1c1 0.29

RNA 3T3 3T3/26 3T3/07 3T3/pw1c1

$\alpha 1$ mouse mouse GAPDH M



[illegible]

PCR

10 X Buffer
 Primers Amp 3/S
 dNTP MIX (15mM, 1994)
 $\alpha^{32}P$ dCTP
 Tag Polymerase (Boehringer
 Hro
 DNA (1/10 of each Dnt)

12 cycles

μ l	X23	
10	230	} 70 μ l / sample
4	92	
1.4	32.2	
1	23	
0.4	9.2	
53.2	1223.6	
30 μ l		

File #32, 37

1/10 of each DNA used in PCR

Amp Primers were used for all DNA quantitation

Thus:

$$\text{Gene Copy No} = \frac{\text{ng read directly from the standard curve}}{2 \times 10^8} \times \frac{1}{\text{Fraction of DNA used in PCR}}$$

total plasmid copy # $\times 10^7$

Rat-1 / 3.6 Kb promoter (Rat)

14

Rat-1 / 2.5 " " (mouse)

270

Rat-1 / 1.0 " " (Rat)

84

Rat-1 / 0.22 " " (mouse)

280

1302 / 3.6

5.6

1302 / 2.5

174

1302 / 1.0

76

1302 / 0.22

266

1-19 / 3.6

24

1-19 / 2.5

90

1-19 / 1.0

54

1-19 / 0.22

208

Rat-1 / BB 0.7

130

Rat-1 / BB 2.6

42

Rat-1302 / BB 2.6

52

3T3 / 0.7

18

3T3 / 2.6

28

3T3 / PWTCL

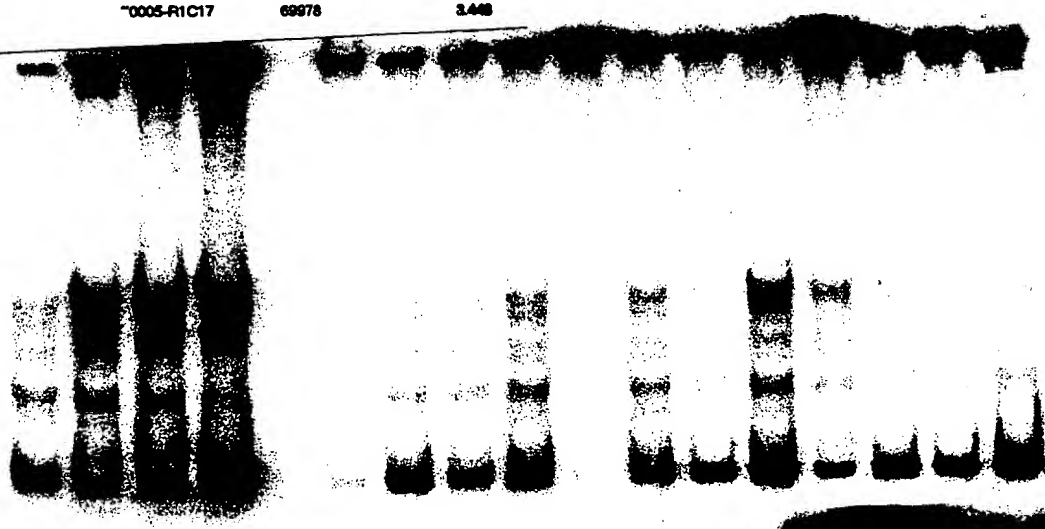
156

titations
run

Standard
Space

OBJECT NAME	VOLUME	BACKGROUND
"0005-R1C1	70562	4.129
"0005-R1C2	283159	5.476
"0005-R1C3	593639	6.532
"0005-R1C4	774686	8.225
"0005-R1C5	20334	4.334
"0005-R1C6	15121	3.961
"0005-R1C7	84978	4.060
"0005-R1C8	41412	3.964
"0005-R1C9	85320	4.061
"0005-R1C10	7861	3.953
"0005-R1C11	65157	4.076
"0005-R1C12	40515	3.799
"0005-R1C13	82279	3.966
"0005-R1C14	19363	3.888
"0005-R1C15	45047	3.888
"0005-R1C16	32617	3.791
"0005-R1C17	69978	3.448

OBJECT NAME	VOLUME	BACKGROUND
"0007-R1C1	14492	3.995
"0007-R1C2	85406	3.932
"0007-R1C3	42231	3.794
"0007-R1C4	87179	3.996
"0007-R1C5	8403	3.975
"0007-R1C6	66214	4.036
"0007-R1C7	39634	3.980
"0007-R1C8	85046	4.032
"0007-R1C9	20156	3.667
"0007-R1C10	44964	3.766
"0007-R1C11	32647	3.732
"0007-R1C12	72990	3.888



OBJECT NAME	VOLUME	BACKGROUND	# PTS IN OBJECT	AVERAGE
"0009-R1C1	54439	3.311	2544	24.71
"0009-R1C2	28585	3.497	2597	14.50
"0009-R1C3	32486	3.423	2597	15.93
"0009-R1C4	18887	3.519	2597	10.05
"0009-R1C5	21682	3.549	2597	12.84
"0009-R1C6	61289	3.578	2544	27.86



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